Osteoblasts and MG-63 osteosarcoma cells behave differently when in contact with ProRoot$^{\text{TM}}$ MTA and White MTA

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Abstract

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Aim To test the hypothesis that MG-63 osteosarcoma cells and primary osteoblasts react differently to ProRoot$^{\text{TM}}$ MTA (mineral trioxide aggregate) and White MTA by: (i) investigating the attachment of primary osteoblasts and MG-63 osteosarcoma cells to ProRoot$^{\text{TM}}$ MTA and White MTA; and (ii) comparing the osteogenic behaviour of both cell lines in contact with these endodontic materials.

Methodology Primary osteoblasts were harvested from foetal rat calvaria by sequential digestion and MG-63 osteosarcoma cells were purchased. Cells were exposed to ProRoot$^{\text{TM}}$ MTA and White MTA prepared according to the manufacturer's instructions. All samples and controls were prepared in quadruplicate. After 6, 9 and 13 days exposure to MTA, the cells were fixed and prepared for SEM examination. In addition, both the cell types were grown to confluence and exposed to β-glycerophosphate and dexamethasone to assess mineralized nodule formation as a function of osteogenic behaviour.

Results The number of cells on the surface of the culture dish and on top of the materials increased in all samples throughout the 3 time periods, except for White MTA where no primary osteoblasts were visible on top of the material by the end of 13 days. After exposing cells to differentiation medium nodules were observed in cultures of primary osteoblasts, but not of MG-63 osteosarcoma cells.

Conclusions Under the conditions of this study, whilst primary osteoblasts initially bound to White MTA, they did not survive on the surface by the end of 13 days. Primary osteoblasts formed mineralized nodules when exposed to differentiation medium, whilst MG-63 cells did not form nodules. As MG-63 cells do not behave osteogenically by forming mineralized nodules, and primary osteoblasts are more sensitive than MG-63 osteosarcoma cells to White MTA in cell culture, primary osteoblasts are more appropriate than MG-63 cells for testing endodontic materials in cell culture.

Keywords: MG-63 osteosarcoma cells, mineral trioxide aggregate, osteoblasts.

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Introduction

Historically, endodontic materials have undergone extensive testing, both *in vivo* and *in vitro*. *In vivo* testing, such as implantation and usage tests, have an advantage in that they allow complex interactions between the host and the material to be examined. *In vitro* tests, such as cell culture, enable experimental factors to be controlled, which often is a significant problem when performing experiments *in vivo* (Spångberg 1989). Therefore, both types of testing are relevant and should be performed when a new material is introduced.

ProRoot$^{\text{TM}}$ MTA (mineral trioxide aggregate; Dentsply Endodontics, Tulsa, OK, USA) is a material that has been used worldwide, with several clinical applications such as apical barriers in teeth with immature apices

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(Shabahang *et al.* 1999, Shabahang & Torabinejad 2000), repair of root perforations (Lee *et al.* 1993), root-end filling (Bates *et al.* 1996, Torabinejad *et al.* 1993, 1995), direct pulp capping (Pitt Ford *et al.* 1996) and pulpotomy (Eidelman *et al.* 2001). This material has been tested thoroughly *in vivo,* through implantation tests (Torabinejad *et al.* 1998) and usage tests (Torabinejad *et al.* 1997). Because this material comes in contact with vital tissues it must be biocompatible and should favour regeneration of the involved tissues to their prediseased status (Torabinejad & Chivian 1999).

MTA may be the ideal material because it is the only material that consistently allows for the regeneration of the periodontal ligament, the apposition of a cementum-like material and formation of bone (Schwartz *et al.* 1999a). With respect to the latter, there are several studies that have examined osteoblast response to ProRoot MTA. Changes in cell morphology and cytokine production in cultured human MG-63 osteosarcoma cells in contact with MTA have been investigated (Koh *et al.* 1998). This is an established cell line, which has an aneuploid chromosome pattern, and cells multiply rapidly with an unlimited life span. MG-63 cells adhered to MTA at 1 and 3 days and levels of interleukins (IL-1α, IL-1β and IL-6) were raised when cells were grown in the presence of MTA (Koh *et al.* 1998). Mitchell *et al.* (1999) investigated the biocompatibility of variants of MTA by culturing MG-63 cells in the presence of variants of this material and observing cytomorphology, cell growth and cytokine expression in these cells. After 2 days the material was almost completely covered by cells, and three variants of MTA induced increased expression of IL-6 and IL-8 over the controls.

ProRoot^{TM} MTA has been associated with the occasional staining of teeth when used in pulpotomy and pulp capping procedures. Therefore, a White MTA material (Dentsply Endodontics, Tulsa, OK, USA) has been developed recently to overcome this concern. As with other endodontic materials, the White MTA should undergo the same extensive testing as the ProRoot^{TM} MTA.

Whilst transformed cell lines have been used, cell cultures of euploid cells have been suggested to test materials that contact the bone *in vivo.* A human osteoblast-like cell culture has been useful to test and classify cell specific toxicity of materials exhibiting low toxicity (Lang & Mertens 1990). However, whether primary diploid cells or aneuploid cell lines should be used when testing materials is controversial (Spångberg 1989).

This study was designed to test the hypothesis that MG-63 osteosarcoma cells and primary osteoblasts react differently to ProRoot^{TM} MTA and White MTA by: (i) Investigating the attachment of primary osteoblasts and MG-63 osteosarcoma cells to ProRoot^{TM} MTA and White MTA; and (ii) comparing the osteogenic behaviour of both cell lines in contact with these endodontic materials.

Materials and methods

Preparation of cell cultures

Primary osteoblasts were harvested from 20-day-old-foetal rat calvaria as described previously (Williams *et al.* 1980). Briefly, Sprague–Dawley timed-pregnant rats (F20) were killed using inhalation Halothane overdose and the uteri were removed surgically and stored on ice. Foetuses were decapitated and the calvaria removed and placed in a dish containing Hank's balanced salt solution (HBSS, Life Technologies; Rockville, MD, USA). The coronal, lambdoid and sagittal sutures were dissected under a light microscope and discarded.

The parietal and frontal bones were cut into small pieces and digested in a 1 : 1 solution of collagenase (Sigma; St. Louis, MO, USA) and phosphate buffered saline (PBS; Sigma, St. Louis, MO, USA). Following agitation for 20 min at 37 °C, the supernatant was removed and new solution was added to the remaining bone chips for a further 20 min. The first two supernatants were discarded and the third, fourth and fifth supernatants were collected and stored on ice. These fractions were combined and centrifuged at 228 *g* for 5 min. The cell pellet was re-suspended in culture medium prepared according to Opperman *et al.* (1995). An antibiotic combination of 76 U mL^{-1} penicillin and 76 μg mL^{-1} streptomycin (Life Technologies; Rockville, MD, USA), was used in place of gentamycin. The medium was supplemented with 10% foetal bovine serum (FBS; Life Technologies; Rockville, MD, USA). Cells were plated in 60 mm dishes and placed in a 5% CO_2 air atmosphere at 37 °C. Bone chips were plated separately in the same culture medium and under the same conditions. Culture medium was changed as needed and the cells were subcultured when they reached confluence, using 0.25% trypsin (Life Technologies, Rockville, MD, USA) in Mg^{++} and Ca^{++} free PBS. Cells through the fourth passage were used in the experimental procedures of this study.

MG-63 human osteosarcoma cells were obtained from American Type Culture Collection (ATCC; Rockville, MD). Immediately upon arrival, cells were thawed and suspended in a 100-mm culture dish with Vitacell's Minimum Essential Medium (EMEM modified, ATCC) supplemented with 10% heat inactivated FBS. The dish was placed in an atmosphere of 5% CO_2 at 37 °C. Cells were subcultured several times when they reached confluence using 0.25% trypsin in Mg^{++} and Ca^{++} free PBS before using them.

Scanning electron microscopic examination

ProRoot™ MTA and White MTA were prepared according to the manufacturer's instructions. Pellets of the material were manufactured under sterile conditions using an MTA carrier. Eight-well slides were used, placing one pellet per well in four of the wells on each slide. The remaining four wells served as controls. A total of 16 slides were prepared, 8 containing ProRoot™ MTA pellets and the other 8 containing White MTA pellets. Immediately after placing the pellets in the wells, primary osteoblasts and MG-63 osteosarcoma cells were seeded into the wells at 10^4 cells per well. Slides were placed at 5% CO_2 in air and 37 °C. After 6, 9 and 13 days slides were fixed with 4% paraformaldehyde (PFA), dehydrated, and after critical point drying, the samples were sputter coated and viewed through the Scanning Electron Microscope (JEOL JSM-6300).

Nodule formation

Primary osteoblasts and MG-63 osteosarcoma cells were passaged into eight 35 mm dishes, four with each cell line. Cells were grown to confluence and then the medium was replaced with differentiation medium, which consisted of normal bone medium as described for each cell line plus 1 mM β-glycerophosphate and 10^{-8}M dexamethasone. Two of the dishes for each cell line were exposed to medium without dexamethasone, thereby acting as a non-mineralizing control. The dishes were kept in an atmosphere of 5% CO_2 at 37 °C and the differentiation medium was changed as needed. At 14, 21 or 23 days, cells were fixed with 4% PFA for 1 h and then washed with ddH_2O thrice. Thereafter, 0.2% Alizarin red was added to each dish for 5 min. The dishes were washed thrice with ddH_2O and the last was left covering the cells. Cells were examined and photographed using a 10× dipping lens on a Zeiss Axiophot microscope.

Results

Scanning electron microscopic examination

Day 6

Healthy cells could be seen in all the control wells of both primary osteoblasts and osteosarcoma cells (not shown). In all the experimental wells, healthy cells could be seen all over the bottom of the plate and all around the materials which were placed in the middle of each well (Fig. 1A,B,C). Some primary osteoblasts and osteosarcoma cells could be observed proliferating on the surface of the materials (Fig. 1B,D).

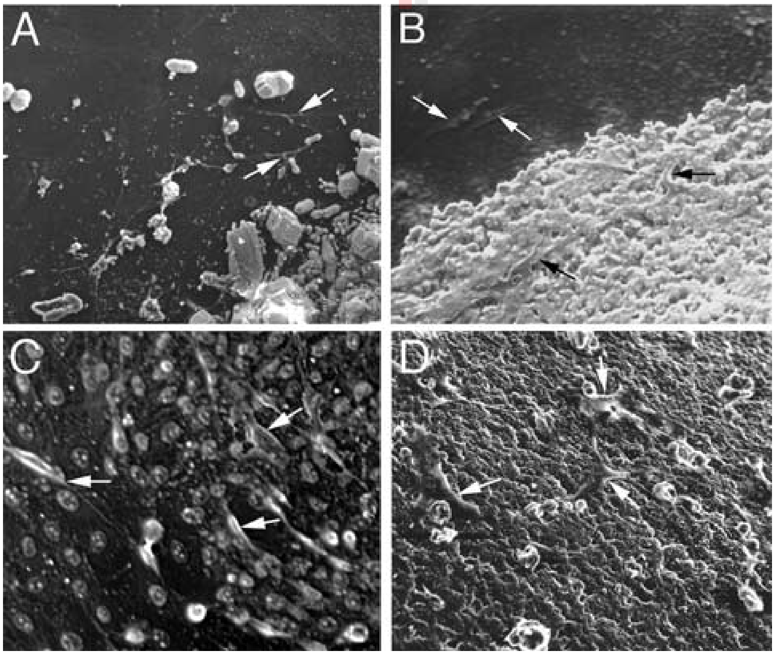

Figure 1 6 days, 350×: (A) ProRoot™ MTA: primary osteoblasts are seen on the bottom of the well between the ProRoot™ MTA particles (arrows); (B) White MTA: primary osteoblasts are seen on the bottom of the well between the White MTA particles (white arrows) and some on the surface of the material (black arrows); (C) ProRoot MTA: MG-63 osteosarcoma cells are seen attached to the bottom of the well between the ProRoot™ MTA particles in the area along the border of the MTA pellet (arrows); (D) White MTA: MG-63 osteosarcoma cells are seen on top of the material (arrows).

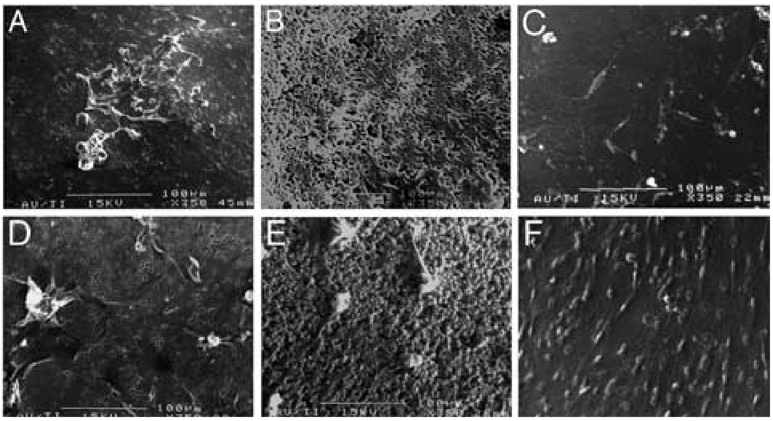

Figure 2 13 days, 350×: (A) ProRoot^{TM} MTA: primary osteoblasts are observed attached to the top of the material; (B) White MTA: primary osteoblasts not seen on top of the material; (C) White MTA: primary osteoblasts could be observed on the dish surrounding the material; (D) ProRoot^{TM} MTA: MG-63 osteosarcoma cells seen on top of the material; (E) White MTA: MG-63 osteosarcoma cells are present on top of the material; (F) osteosarcoma cells can be seen 100% confluent on the bottom of the control dishes without materials.

Day 9

Osteosarcoma cells and primary osteoblasts in all the control wells were confluent on the bottom of the dish (not shown). In all the wells containing the experimental materials, osteosarcoma cells and primary osteoblasts could be seen on top of both the ProRoot^{TM} and the White MTA (not shown).

Day 13

Osteosarcoma cells were observed in large quantities on top of both materials in all the wells (Figs 2D,E and 3A,B). However, primary osteoblasts could only be seen on top of the ProRoot^{TM} MTA (Fig. 2A). Whilst primary osteoblasts could be observed on the dish surrounding the White MTA (Fig. 2C), no cells could be seen on the material itself (Fig. 2B). Osteosarcoma cells can be seen 100% confluent on the bottom of the control dishes without materials (Fig. 2F). Primary osteoblasts in the control wells were confluent, similar to the osteosarcoma cells (not shown).

Nodule formation

Primary Osteoblasts

Cells were fixed at 21, 25 and 28 days. Nodules were formed only by cells exposed to dexamethasone. In control dishes no nodules were found (Fig. 4).

MG-63 osteosarcoma cells

At day 14 one dish with dexamethasone was stopped. The remaining two dishes containing dexamethasone were stopped at day 23. No nodules were found at either time period (Fig. 5).

Discussion

In the present study, two different osteoblast cell lines were evaluated morphologically to characterize their behaviour when in contact with ProRoot^{TM} and White MTA. Under the conditions of this study, MG-63 osteosarcoma cells adhered to White MTA for periods twice as

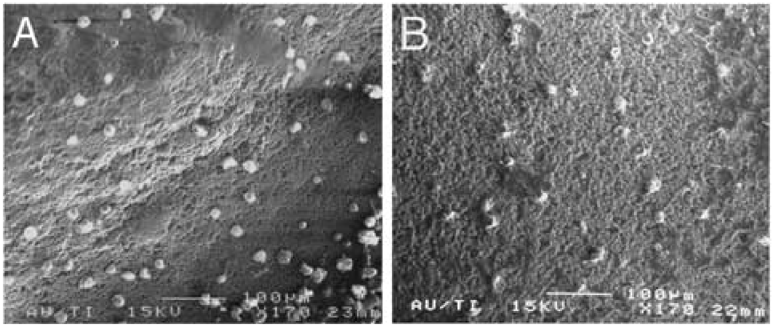

Figure 3 13 days, 170×: (A) White MTA: MG-63 osteosarcoma cells on top of the material; (B) ProRoot^{TM} MTA: MG-63 osteosarcoma cells on top of the material.

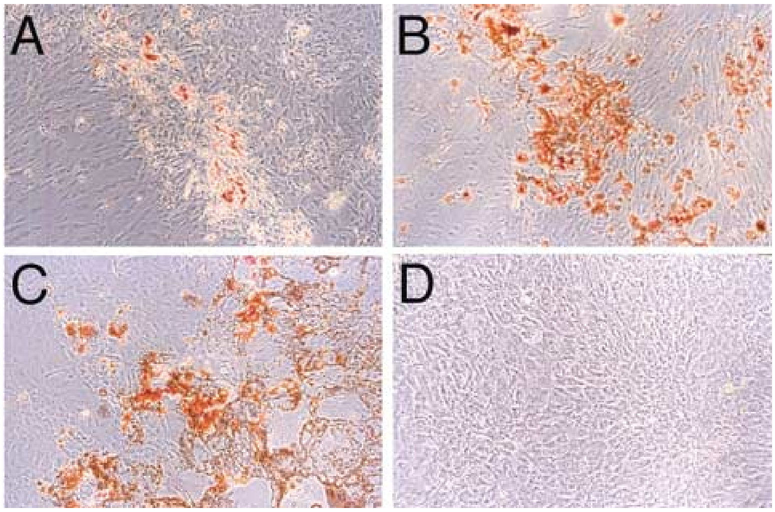

Figure 4 (A) Primary osteoblasts with dexamethasone 10×, 21 days; (B) primary osteoblasts with dexamethasone 10×, 25 days; (C) primary osteoblasts with dexamethasone 10×, 28 days; (D) control, primary osteoblasts without dexamethasone 10×, 21 days. Mineralized nodules are stained positively with Alizarin red.

long as primary osteoblasts. There were no differences between cell lines in their adherence to ProRoot^{TM} MTA.

Osteoblasts are anchorage dependent cells that must adhere to their substrate in order to be active. Cell adhesion is a complex and dynamic process that plays a critical role in wound healing and has implications in cell growth, proliferation and differentiation. Primary cells must adhere to matrix proteins to survive and proliferate. When these cells are cultured under conditions that prevent adhesion and spreading, they stop growing and lose viability. This is in contrast to transformed cells that may be anchorage-independent, enabling tumour cells *in vivo* to survive and metastasize (Mashima & Mizuguchi 1992, Re *et al.* 1994, Fukazawa *et al.* 1995).

The lack of attachment of primary osteoblasts to White MTA for longer periods of time could be related to differences in surface roughness or in the chemical composition of the materials. The roughness and topography of a surface is what dictates the type of focal attachment that can occur. There are several studies that have investigated the role of surface roughness in the osteogenic response (Boyan *et al.* 1999, Schwartz *et al.* 1999b). Surface topography has a direct impact not only on the attachment, but also on migration, proliferation and differentiation of osteoblasts. A roughness greater than the cell length would be perceived as smooth by those cells located between adjacent peaks. In this case, the cell would be able to attach and spread in a similar way as it does on tissue culture plastic. As surfaces become rougher, proliferation of the cells decreases and their activity as mature secretory osteoblasts increases. According to the manufacturers, there is very little difference in the particle size of ProRoot^{TM} and White MTA. However, when hardened these two materials have very different surface topographies when viewed by SEM (Fig. 2). Furthermore, there is a significant reduction in the iron component of the White MTA as compared to the original ProRoot^{TM} MTA formulation that could contribute to the osteoblasts not remaining attached to the White MTA.

Unfortunately, SEM evaluation did not permit collection of numeric data and consequently any statistical analysis in this study. Previous studies that have investigated cell attachment to MTA have reported descriptive data as well (Koh *et al.* 1997, Koh *et al.* 1998, Zhu *et al.* 2000). Mitchell *et al.* (1999), in a similar study, used a scoring system but were not able to do any statistical analysis owing to insufficient data.

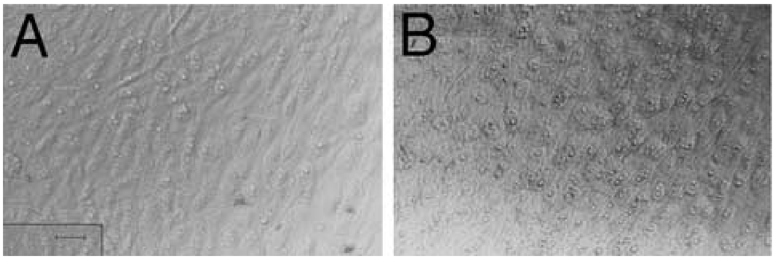

Figure 5 (A) MG-63 osteosarcoma cells with dexamethasone 10×, 14 days; (B) MG-63 osteosarcoma cells with dexamethasone 10×, 23 days. Scale bar: 60 μm.

Whether primary diploid cells or aneuploid cell lines should be used when testing materials is controversial (Lang & Mertens 1990, Clover & Gowen 1994, Koh *et al.* 1998, Tai *et al.* 2001). Primary osteoblasts have a diploid chromosome pattern, are characterized by growing slowly and have a finite lifespan. Established cell lines, on the other hand, have an aneuploid chromosome pattern, tend to multiply rapidly and, if appropriately subcultured, have unlimited lifespan (Spångberg 1989, Freshney 1994). An advantage of using a permanent cell line is that it provides phenotypically consistent and stable cell populations, large enough for biochemical analyses. However, it may not always be possible to extrapolate results from osteosarcoma cell cultures because immortalization can affect cellular behaviour.

Established laboratory cell lines have been used in previous studies investigating cell morphology in the presence of MTA (Koh *et al.* 1997, Koh *et al.* 1998, Mitchell *et al.* 1999, Zhu *et al.* 2000). In the present study, an established cell line (MG-63) and rat primary osteoblasts were used. Primary cell strains derived from living tissues are necessary and have been recommended by the ISO for specific sensitivity testing to simulate the *in vivo* situation (Schmalz 1994, Keiser *et al.* 2000).

In this study, primary osteoblasts were obtained from foetal rat calvaria. This is an excellent source of osteoblasts because cells from young animals proliferate rapidly. Cells from the third, fourth and fifth digests were collected because these later digests provide a more pure culture, containing mostly cells that express an osteoblast-like phenotype (Luben *et al.* 1976). The osteoblast phenotype of the cells isolated for this study was determined by the production of organized mineralized nodules by the cells. This is in agreement with Bellows *et al.* 1991) who found nodules in rat calvaria cells that were grown in a medium containing ascorbic acid and dexamethasone as early as 17 days. MG-63 osteosarcoma cells were cultured under the same conditions as the primary osteoblasts, but no nodules were observed, as has been shown previously (Schwartz *et al.* 1999b). Primary osteoblasts are therefore able to differentiate in culture, whilst MG-63 cells do not. This could account for the differences seen in extended attachment of these cells to MTA. It is possible that both MG-63 cells and primary osteoblasts can adhere to and divide on both materials. However, as osteoblasts begin to differentiate, they no longer are able to remain attached to White MTA.

Under the conditions of this study, primary osteoblasts were more sensitive than MG-63 osteosarcoma cells to White MTA in cell culture. Therefore, primary cells should be used for sensitivity testing of endodontic materials. This is in agreement with Schmalz (1994) who states that primary cells provide good simulation of the *in vivo* situation, thus being indicated for specific scientific problems.

As such different results were found when transformed versus primary cell lines were used, it is critical to determine whether these differences result from differences in cell viability, cell proliferation or cell migration. These studies are currently under way. Whilst no immediate clinical relevance can be drawn from these *in vitro* studies, it is important for the clinician to note that the characteristics of these two materials differ sufficiently to elicit different cellular responses. The importance of these differences in a clinical setting should be established by in depth *in vivo* studies.

Conclusions

Under the conditions of this study, primary osteoblasts did not remain attached to the surface of White MTA in long-term culture. These findings merit further investigations.

As primary osteoblasts were more sensitive than MG-63 osteosarcoma cells to White MTA in cell culture, these cells are more appropriate to test endodontic materials *in vitro*.

Finally, tissue culture studies are one of many tests, such as implantation and usage tests, which are used prior to determining recommended usage parameters for any given product or material formulation.

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